Bryant University

HONORS THESIS

Biochemical Characterization of Diamide Inhibitors with N-acetylglucosaminidases LytG from Bacillus subtilis

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Submitted in partial fulfillment of the requirements for graduation
with honors in the Bryant University Honors Program

APRIL 2016

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Biochemical Characterization of Diamide Inhibitors with N-acetylglucosaminidases LytG from *Bacillus subtilis*)
Senior Capstone Project for Drew Phelan

ABSTRACT

In recent years the frequency of antibiotic resistance has been on the rise creating a need for antibiotic development with specific and lethal targets. It has been recently reported that glycosyl trizole are a novel class of antibacterial agents (1). Further investigation on the antibacterial ability of glycosyl triazole inhibitors has shown that targets include *exo*-acting N-acetylglucosaminidases (GlcNAcase) LytG (*Bacillus subtilis*) and FlgJ (*Salmonella enterica*) of the GH73 family (2). The Glycoside Hydrolase Family 73 (GH73) is characterized by bacterial and viral glycoside hydrolase. This enzyme cleaves the β-1,4-glycosidic linkage between *N*-acetylglucosaminyl (NAG) and *N*-acetylmuramyl (NAM) of the carbohydrate backbone in bacterial peptidoglycan. Glycoside hydrolase can occur as an endo- or exo- process, depending on the region of the chain that is cleaved. Endo-acting refers to activity in the middle of the chain, whereas *exo*-acting refers to the ends (typically the non-reducing end) (3). Currently, there is no kinetic parameters that have been determined for any member of the GH73 family, however binding and kinetic characterization will be performed for select glycosyl triazole inhibitors and GH73 targets interactions. Further studies will involve crystallization and GlcNAcase activity assays to identify GH73 family members as the target of glycosyl triazole inhibitors. Through these studies the interaction between the non-competing inhibitor and the GH73 target will be characterized. Additionally, it will be demonstrated that these Ugi- derived compounds are competitive inhibitors of GH73 enzymes.

INTRODUCTION

Peptidoglycan is one of the most important elements of the cell that protects its' integrity and shape as well as maintains its electrolyte balance and confer stability and strength. When the peptidoglycan layer within the cell is inhibited or interfered with the cell may even signal certain pathways that will initiate apoptosis. Being such an integral part of the cells ability to survive, peptidoglycan serves as an ideal target for antibiotic compounds. While many have been clinically successful with exploiting this target (including vancomycin and β-lactams), one target in particular has been left unexploited- N-acetylglucosaminidase (GlcNAcases). This bacterial autolysins is an important element, responsible for making space for incorporating new material into the cell as well as assisting in cell division which may include vegetative growth, daughter cell separation or hydrolysis of the septum upon separation (5). Since GlcNAcase is such a vital aspect of the cell, we chose it as our target. Preliminary data has resulted in the filing of a provisional patent (1) which encompasses the use of diamide inhibitors against Gram-positive bacteria. The widespread and inappropriate use of antibiotics have rendered the world almost powerless against antibiotic resistant strains and the newest 'superbugs'. Every year in the United States alone antibiotic resistant strains cause 23,000 deaths and an estimated $20 billion in additional health care cost (6).

LITERATURE REVIEW

Gram Staining

Through the process of Gram staining, bacterial species can be differentiated into the two groups of Gram- negative and Gram-positive. Differing these two categories is the characteristics of the layers peptidoglycan within the cell wall, capitalizing of this differentiation, Gram- staining detects peptidoglycan. This procedure begins with the air-dried, fixed bacterial smears being stained with crystal violet stain and rinsed which turns both Gram-positive and Gram- negative cells purple. Additionally, an iodine solution is then added to the smear and rinsed which turns both cell types a blue-purple. Next, an alcohol wash is added which is what begins differentiating Gram-positive from Gram- negative bacteria. The Gram-positive cells contain a thick layer of peptidoglycan which resists the effects of the alcohol wash and maintains the blue-purple coloring. Contrastingly, Gram-

negative cells have a much thinner layer of peptidoglycan and losses the blue-purple staining. Finally, the last step is the addition of Safranin, an orange- red stain. This is picked up by the Gram- negative bacteria, but again Gram-positive bacteria resists the coloring and remains blue-purple (7). This staining technique is an empirical method that relies solely on the detection of the outer lipid membrane (8).

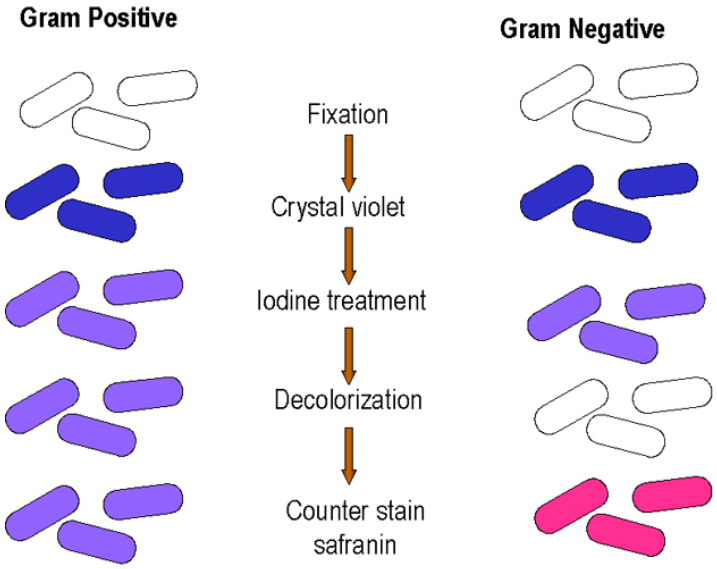


Figure 1 : This image depicts the process of Gram staining and the subsequent coloring that results in both Gram- positive and Gram- negative species at each step of the procedure(9).

Gram-negative and Gram-positive Bacteria

Gram – negative bacteria consist of a thin layer of peptidoglycan that is paired with an outer membrane with lipopolysaccharide (LPS) which contains lipid A, a core polysaccharide and an O antigen**.** LPS, an endotoxin, and is a major facet to the structural integrity of bacteria and the protection of the cell membrane**.** In Gram- negative bacteria this component of the cell wall is absolutely vital to the life of the cell and if this structure is destroyed or mutated the result would be cell death**.** Further differentiating the Gram-positive from the Gram-negative is the presence of an outer membrane and the relative thinness of the cell wall (ranging from only 8-12nm thick compared to Gram- positive bacteria which ranges closer to 20-30nm thick)**.** The cell wall of Gram-negative bacteria is also wavy and is only tethered to the plasma membrane at a select few locations, rather than tightly bound and smooth in Gram-positive

bacteria (10). Additionally, unique to Gram-negative bacteria is the porins that are present in the outer membrane. These porins are beta barrel proteins that channel through the plasma membrane and act as pores allowing for passive diffusion of water, ions and some small molecules (10). Finally, the absence of teichoic acid within Gram-negative species differentiates from the Gram-positive cell walls in which it is present within the peptidoglycan layer (11). It should be noted that these characteristics are mere guidelines and have exceptions.

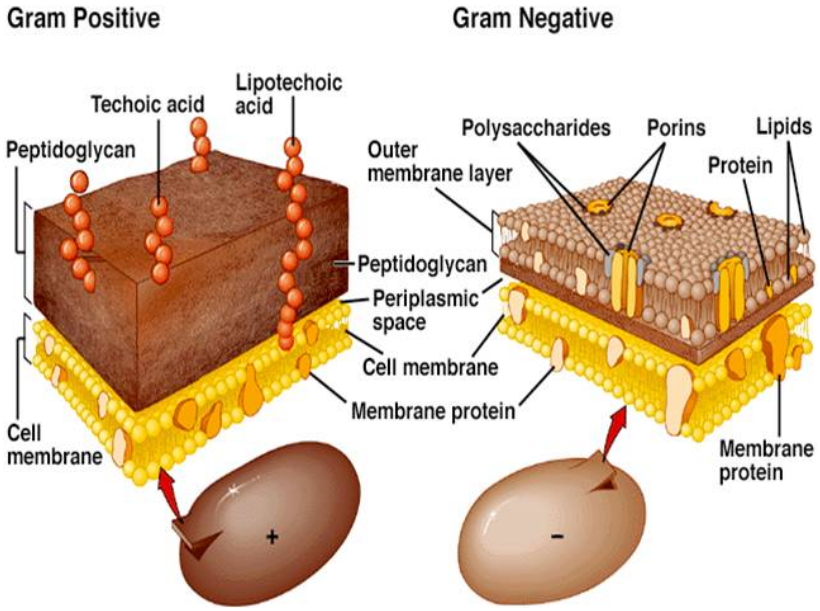


Figure 2: This image demonstrates the physical structural differences between Gram- positive and Gram- negative bacterial cell wall including the presence (or absence) of techoic acid, lipotechoic acid and porins (12).

<u>*Bacillus subtilis*</u>

Bacillus subtilis (B. subtilis) is commonly found in soil, water and the gastrointestinal tracts of various organisms including humans. In severely immune-compromised individuals, the presence of *B. subtilis* can cause harmful effects, contrastingly it can also be used as a probiotic in individuals who are not immune-compromised (13). *B. subtilis* is extremely resistant to heat exposure which is evident through the plethora of strains that are found in

spoiled bread known as 'roping' which results in the sticky production of long- chain polysaccharides due to *B. subtilis* (14). *B. subtilis* is also a facultative aerobe, meaning that it can grow and survive in both aerobic and anaerobic environments. In anaerobic instances, the cell will use nitrite or nitrate as the terminal electron acceptor instead of oxygen. This catalase positive species converts hydrogen peroxide into components that won't have the damaging effects of hydrogen peroxide has on cellular metabolic process.

The use of flagella allows for cells to swarm on surfaces, but not liquids. When *B. subtilis* connects with neighboring cells they lose their ability to move individually and thus must move as a cohort, referred to as a 'raft'. A slime layer containing surfactin, a lipopeptide that is able to reduce surface tension, must first be secreted before the *B. subtilis* rafts are able to move (15).

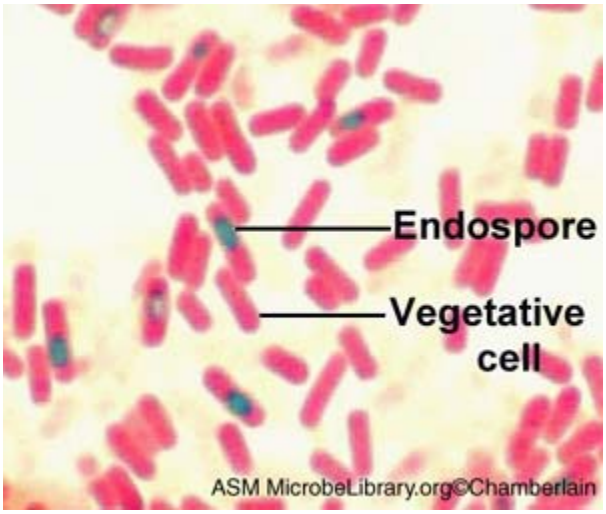


Figure 3: Shown here is a micrograph of Bacillus subtilis containing both vegetative cells and endospores (16).

B. subtilis is viewed as an exemplary model to study fundamental properties and characteristics of typical Gram-positive spore- forming bacteria. *B. subtilis* is able to ferment quickly with high product yield which also makes it an ideal organism to work with. Endospore formation studies also frequently use *B. subtilis* as the model organism since they

are found mostly in the tips of protuberances. Particularly helpful in cellular development and differentiation experimentation, *B. subtilis* will also be an ideal organism for studying GlcNAcase due to its indirect involvement in the cell division. The ease of genetic mutation that is associated with *B. subtilis* also promotes it as a model organism for laboratory work.

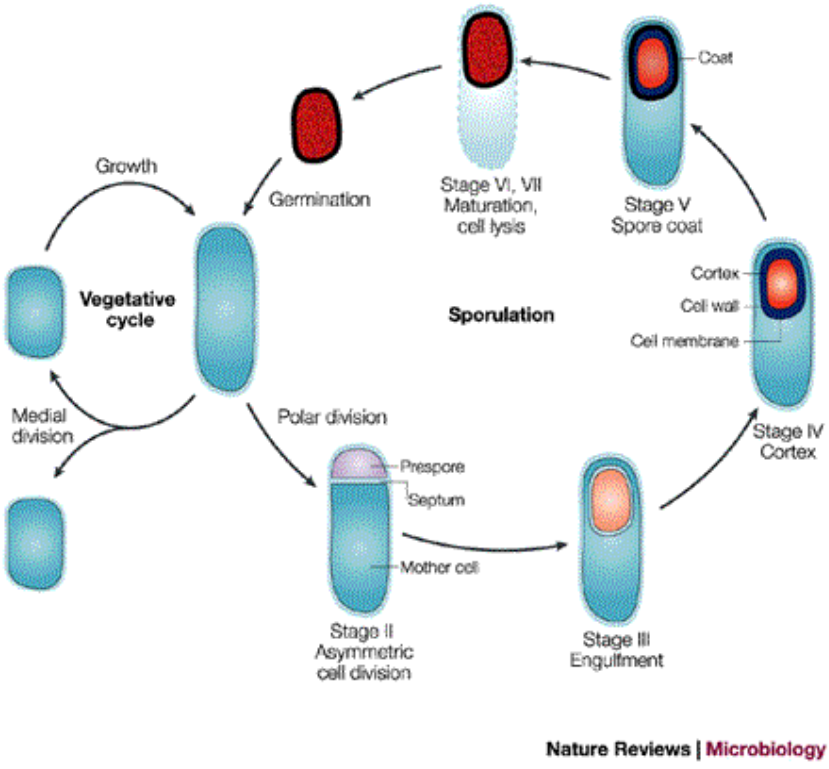


Figure 4: During the vegetative cycle the B. subtilis cell medially divides and grow; next the cell regulator determines if it remains in the vegetative cycle or proceeds into the sporulation cycle. This cycle begins with asymmetric cell division with the formation of a relatively smaller prespore and a mother cell. The mother cell then engulfs the prespore, deposits a spore coat and the mother cell lyses. This releases the spore and it then can germinate in favorable environments which brings the cell back into the vegetative cycle (17).

Peptidoglycan in Gram-positive Bacteria

In Gram-positive bacteria this layer of peptidoglycan is thick can often be anywhere from 15-80 nanometers, compared to the relatively thin layer found in Gram-negative bacteria, which

typically consists of a single layer about 10 nanometers thick. Gram-negative peptidoglycan is formed with a carbohydrate backbone that alternates N-acetylglucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc); together these amino acid sugars are linked by β-(1,4)-glycosidic bond. The 3- carbon in MurNAc is substituted for a lactyl ether group, which is derived from pyruvate, which then is able to connect the glycan backbone to a peptide side chain. This peptide side chain contains L-alanine, D-glutamate, Diaminopimelic acid and D-alanine in which D-glutamate, D-alanine and Diaminopimelic acid, as well as MurNAc, are only found in bacterial cells. Neighboring peptide side chains are able to link together through an interpeptide bond between Diaminopimelic acid and D-alanine due to the free amino group on Diaminopimelic acid and the free carboxy group on D-alanine (18). This allows for the strands of peptidoglycan to connect, forming a continuous layer around the cell.
Gram-positive peptidoglycan structure can vary, however the most common composition (found in *S. aureus* and *B. subtilus*) consists of diamino acid instead of Diaminopimelic acid and L-lysine; instead of an interpeptide bond, there is an interpeptide bridge of amino acids. This bridge links the free amino group found on the L-lysine with the free carboxy group, again, found on the D-alanine. It has been found that this interpeptide bridge allows for a higher frequency of cross-bonding between neighboring peptide side chains. Specifically, *S. aureus* consists of an interpeptide bridge with a 5 glycine molecule, referred to a pentaglycine bridge (18).

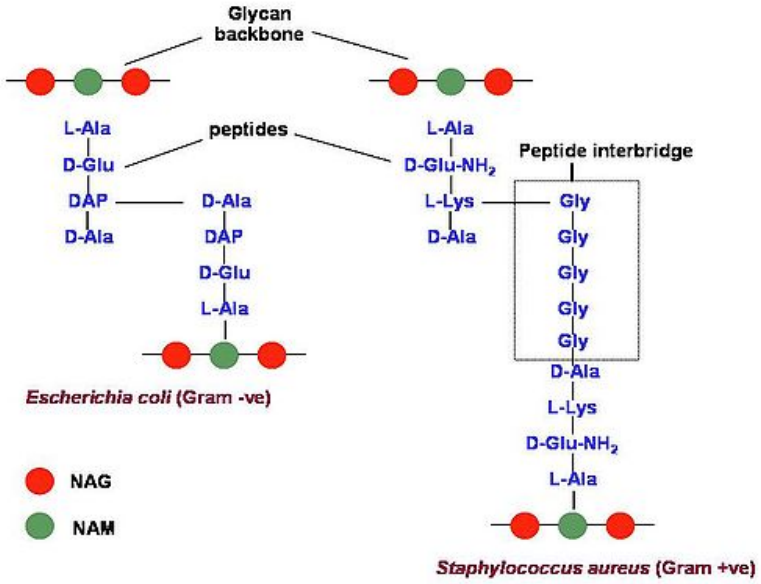


Figure 5: This animation demonstrates the fundamental differences between the peptidoglycan structure of Gram- negative and Gram- positive organisms based on the composition of peptides(19).

Teichoic Acid

Teichoic acid is tightly associated with the formation of the peptidoglycan layer in Gram-positive species. Consisting of polyglycerol or polyribitol with phosphate substitutes and amino acids sugars, Teichoic acid's exact function still remains unknown. It has been proposed that they regulate and mediate positively charged substances through the network of peptidoglycan layers by providing negatively charged channels. Since Teichoic acid is known to occasionally attach to the plasma membrane and protrude into the layers of peptidoglycan, forming lipoteichoic acid, this theory holds substantial merit. Further, it is suggested that they regulate and assemble murmic acid to the outside of the plasma membrane, thereby able to assist in the adherence to tissue surfaces.

Peptidoglycan Biosynthesis

It has also been found that antibiotics targeting the biosynthesis of peptidoglycan, particularly the assembly of interpeptide bridge and bond, is much more efficient in Gram-positive bacteria, compared to Gram-negative. This can be explained by the generally higher

abundance of peptidoglycan in Gram-positive bacteria, as well as the lack of the outer membrane to protect the mesh- layer.

UDP-N-acetyl muramic acid, the precursor subunit of peptidoglycan is synthesized in the cytoplasm, by adding a lactyl group to the 3- carbon of UDP-N-acetyl- glucosamine, UDP-N-acetyl muramic acid is formed. From there five amino acids are added, sequentially, with enzymes rather than t-RNA, to the UDP-N-acetyl muramic acid which forms the pentapeptide side chain, characteristic of *S. aureus*. In *E. coli* L-alanine is followed by D-glutamic acid, meso-diaminopimelic acid, D-alanyl and D-alanine, however this order varies.

The NAM-peptide (pentapeptide or tripeptide) is then transferred from the UDP to bactoprenol phosphate which is sitting on the membrane surface. NAG is then transferred from UDP-NAG to bactoprenol-NAM-peptide which forms the final disaccharide-peptide precursor. The complete NAM and NAG precursor is further transported by the bactoprenol carrier lipid across the plasma membrane to the outer surface. This new disaccharide-peptide is brought to the growing end of the peptidoglycan chain which lengthens it by a chain length. As he bactoprenol pyrophosphate makes its way back through the plasma membrane into the cytoplasm, one phosphate is removed by the enzyme, pyrophosphatase, which leaves the bactoprenol-phosphate able to accept another NAM-peptide to continue the process (20,21). The last step of this process is the linkage of the peptide side chains by either an interpeptide bond or bridge, depending on Gram-negative or Gram-positive.

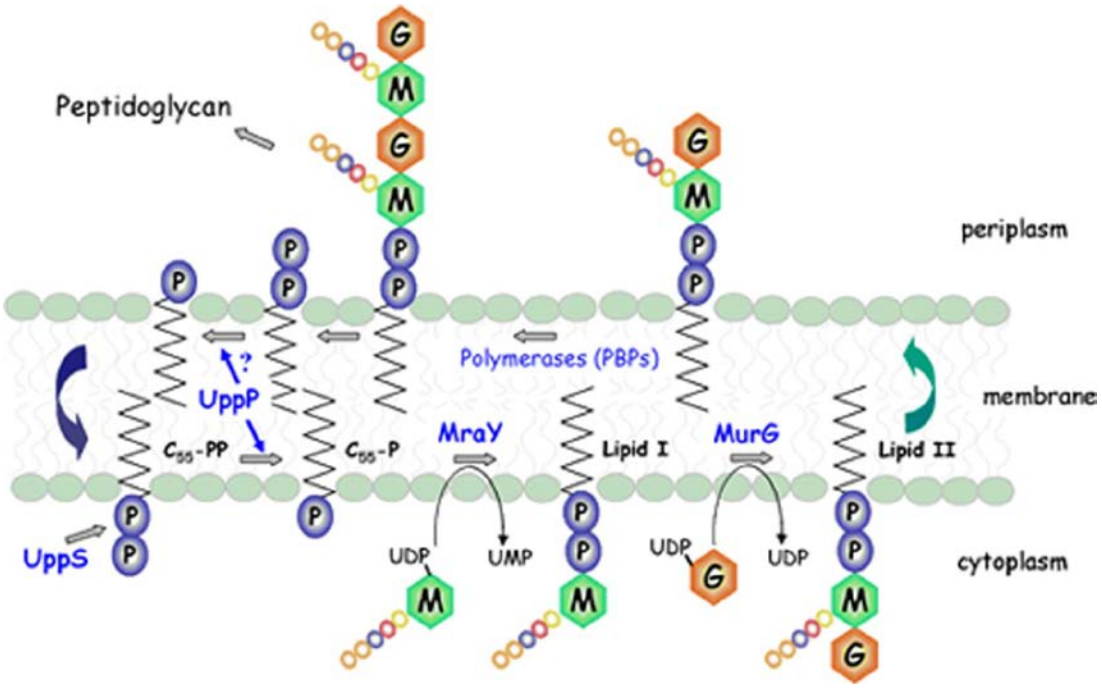


Figure 6: Demonstrating the mechanisms and process of peptidoglycan biosynthesis (22).

Peptidoglycan Function

Functionally, peptidoglycan is responsible for the structural integrity and shape of the cell. A cell is often placed in an environment with a varying osmotic pressure that can be hypertonic, hypotonic or isotonic at times (23). The osmotic pressure is the necessary pressure in order to prevent inward water flow across the membrane. Turgidity is the measure of the turgor pressure, or the pressure that pushes the plasma membrane to the cell wall. When the cell is in a hypertonic solution this means that the levels of solute are higher within the cell than the environment so water flows out of the cell, thus shrinking and shriveling, losing turgidity. A hypotonic solution is the opposite in which water flows into the cell, expanding it and increasing the turgidity. Isotonic solutions are the middle ground between hypertonic and hypotonic and allows for consistent turgidity as the rate or water flowing out is equal of that flowing in. This varying environment can be extreme at times, causing osmotic lysis, or cytolysis in hypotonic environment resulting in a bursting of the cell. The disaccharide

polymer (glycan) being cross-linked with peptides makes it a flexible by strong net-like membrane, thus making it crucial in the maintenance of osmotic pressure for cell integrity. Further, peptidoglycan is related to diffusion of select molecules into the cell, regulation of cell division, communication between cells and is covalently linked to teichoic acid (in Gram-positive bacteria) and other proteins and polysaccharides (24).

During cell division, autolysins are critical in order to break the peptide cross links in peptidoglycan allowing for binary fission to occur and the cell to split into two. These enzymes are followed by transglycosylase enzymes which function to insert new peptide monomers into the holes. Finally, transpeptidase enzymes reinforce the peptidoglycan by reforming the peptide cross- links in the rows.

Antibiotic Resistance

Penicillin works at this last step and blocks transpeptidase from forming the peptide cross links resulting in a weakened peptidoglycan layer which leads to cytolysis when the cell encounters a hypotonic environment (25,26). Very soon after Penicillan's discovery by Dr. Alexander Fleming in 1928 did antibiotic resistance appear. Since, antibiotic resistances has been on the rise due to the poor protocols and habits that have formed since antibiotics introduction.

With emerging multi- drug resistant bacterial strains and a broad geographical location affected have led to a new focus on drug development that prevents inducing bacterial resistance (27). Antibiotic resistance has been linked to inappropriate use, inadequate diagnosis, hospital and agricultural use. Often times, healthcare providers prescribe antibiotics to their patients with a viral- like infection in hopes of eliciting a placebo effect. Often times the antibiotic regime is not completed by the patient or outdated antibiotics are self- prescribed. Both these instances lead to a weakened bacteriostatic impact and an increased frequency of resistance. The heavy use of antimicrobial in elderly, infant, immunosuppressed and ill patients also increases the frequency of antibacterial resistance as

these antimicrobials select for the resistant bacteria, paired with the close quarters of patients in elderly homes, hospitals and prisons, this provides an ideal environment for antibacterial resistant bacteria to spread (28). Finally, the use of antibiotics in agricultural feed accelerates resistance and in fact, more than half of the antibiotics that are created in the United States are used for agricultural purposes (29).

Antibiotic resistance occurs naturally when selected organisms are able to withstand the bacteriostatic characteristics of the antibiotic better than the susceptible cells which are killed or inhibited, a fundamental Darwinian process. This results in selective pressure, the influence that is exerted by antibiotics on the natural selection which promotes the resistant organisms over the susceptible organisms (30).

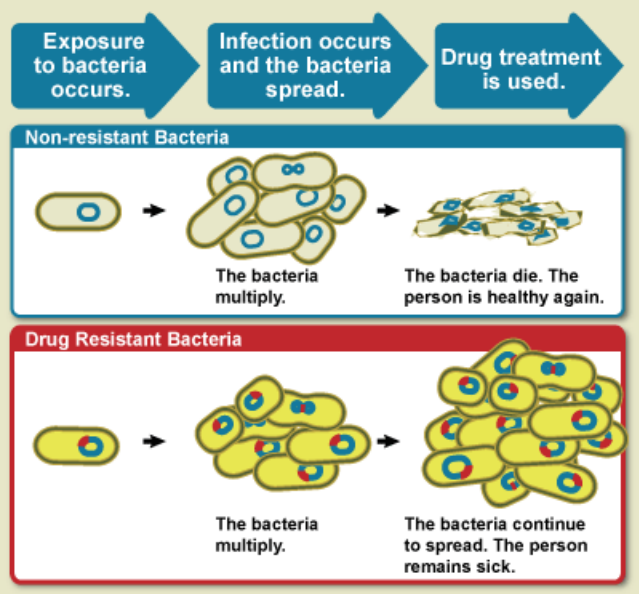


Figure 7: Through the fundamental Darwanian process, antibiotic resistant strains of bacteria are selected and multiplied (31).

Resistance can occur through one of two ways, genetic mutation or acquiring resistance from other bacterium. Genetic mutations are rare and spontaneous changes that occurs when non-resistant bacteria multiplies by the billions. It is estimated that only one million out of every ten million cells are genetically mutated (32) which results in 100,000,000 mutated organisms in a sample size of one billion. These mutated organisms are

selected for as they are resistant to the antibiotics, they multiply and the antibiotic resistant strain of bacteria is now thriving and spreading (33).

Bacteria is also able to acquire antibiotic resistance from neighboring bacterium through Horizontal Gene Transfer (HGT) which is achieved through three mechanisms: transformation, conjugation and transduction. Transformation is the uptake of DNA from the environment, though competency is necessary in order to do so. Some bacteria are able to have competency induced through the addition of calcium chloride which pokes holes in the membrane, or electroporation which delivers electric shocks creating an influx of ions allowing for material (such as are subsequently sucked into the cell. Conjugation is the process in which a cell- to – cell bridge is formed, referred to as a sex pilus which mediates the exchange of F+/- plasmids. After the sex pilus is formed connecting the F+ (F plasmid, male) to the F- (no F plasmid, female), the F plasmid in the male is snipped at the Origin of Transfer (OriT) where relaxosomes then accompany the F plasmid, carrying antibiotic resistant DNA, across the sex pilus. This now transforms the F- into a F+ and allows for the transformed F+ to incorporate the antibiotic resistant DNA into its chromosomal DNA.

The third and final HGT mechanism, transduction, utilizes viruses as the vector for chromosomal DNA fragment transfer. During the lytic cycle of the virus the host chromosome is destroyed and the virus replicates themselves, subsequently killing the cell, however, as the virus enters the lysogenic cycle, temperate phages integrate their DNA into the host chromosome as a prophage. Generalized transduction allows for the phage DNA to be immediately incorporated into the fragmented host chromosomes, fully virulent; DNA fragments are accidently packaged into phages which results in defective phages. After the cell lyses, transduction occurs only if the defective phage interacts with another cell and the host DNA is then integrated and the process continues. In specialized transduction the host chromosome is not fragmented, but instead cut in one place. After the prophage is extracted

from the host chromosomes and replicated, the new phage contains host DNA and is able to interact with neighboring cells to continue the process.

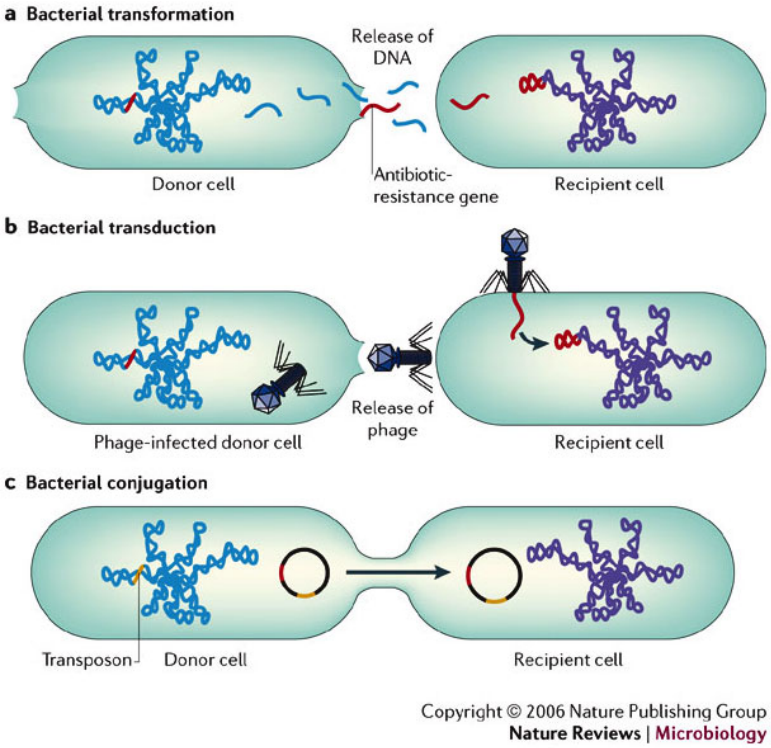


Figure 8: Horizontal Gene Transfer can occur in one of three ways depicted here (34).

There are several variations of antibiotic resistance and their mechanisms widely contrast. The three most common mechanisms of antibiotic resistance are chemical modification of the antibiotic, modification of target site or removal of the antibiotic, yielding it ineffective. The chemical modification works with an enzyme that is specific to the bacteria and antibiotic, such as in the case of Streptomycin which is an antibiotic that targets Tuberculosis infections. Streptomycin binds to the small subunit rRNA which blocks the EF-G activity which is responsible for translocation, thereby inhibiting the ribosomes from being shifted into the P site in order to free up the A site for further protein synthesis (35). The next mechanism involves enzymes that degrades the antibiotic until it is inactive. In Penicillin the enzymes penicillinase are a class of β- lactamase enzymes which cleave the β- lactam ring that is found in Penicillin. Finally, the last mechanism of antibiotic resistance includes the

physical removal of the antibiotic from the cell. Efflux pumps are channels that actively export antibacterial out of the cell utilizing ATP as an energy source or the concentration gradient of hydrogen or sodium ions. This prevents the minimum inhibitory concentration (MIC) to be reached which is necessary in order for the antibiotic to exhibit visible growth inhibition after an overnight incubation (36). The use of an efflux pump is common in Tetracycline, an antibiotic that combats a broad range of ailments from acne, urinary tract infections, gonorrhea and chlamydia.

With the rapidly growing antibiotic resistance problem we are facing, there has been an urgent need for novel antibiotics, as well as for mechanisms that reverse the antibiotic resistance. Restoring antibiotic efficiency has been achieved with slow success through the use of temperate phages to introduce genes that confer sensitivity. Streptomycin and nalidixic acid has achieved success with the introduction of *rpsL* and *gyrA* genes relying on the phage's ability to deliver these genes, rather than the phage's ability to kill the pathogens within the host. This suggests that bacteria will be sensitive to the antibiotics (streptomycin and nalidixic acid) before the host exhibits the infection (36).

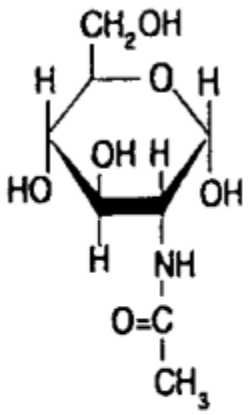
While antibiotic resistance has plague bacteriostatic attempts, it has also burdened our society with social and economic effects. The Centers for Disease Control and Prevention estimates that in the United States $20 billion a year is spent on the healthcare effects of antibiotic resistance and $35 billion a year on societal costs. Antibiotic resistance is also estimated to have caused more than 8 million additional days of being admitted to the hospital in 2011 alone and infected more than 2,049,442 people with 23,000 deaths (37).

Peptidoglycan is vital to the integrity and shape of the cell, thus is often the target for many antibiotics. There have been antibiotics that target the biosynthesis of peptidoglycan, however this method is prone to antibiotic resistance. An ideal target is stable, selectively toxic and not likely to induce bacterial resistance, such as the enzymes that work on the invariant glycan backbone of peptidoglycan, rather than the biosynthesis (38). N-

Acetylglucosaminidases (GlcNAcases) is responsible, in part, for the remodeling and recycling of peptidoglycan, therefore inhibiting GlcNAcase could yield a new spectrum of antibiotics.

GlcNAcase

GlcNAcase are a class of autolysins which are involved in cell division, motility and the assembly of macromolecules (1). GlcNAcases are broken up further into endo-glycosidases or exo-glycosidases. Endo-glycosidases are enzymes that cleave the inner part of sugar chains with a glycon specificity to entire glycan groups of the sugar chain, such as LytD in *Bacillus subtilis.* Contrastingly, exo-glycosidases create small disaccharide products by cleaving the monosaccharide that is located on the non- reducing terminal end of the sugar chain with, found in LytG in *Bacillus subtillus.*



N-Acetylglucosamine

Figure 9: Pictured here is the composition of GlcNAc (39).

Interestingly, since exo-glycosidases have such high glycan specificity, they are ideal targets for structural sugar studies, however the use of *p*-nitrophenyl glycosidase as a substrate does not accurately mimic the structure since it is unable to hydrolyze *p*-nitrophenylglycosides. Exoglycosidase activity can be accurately measured, however, with this method (40).

GlcNAc is a monosaccharide derived from glucose, when alternated with N-acetylmuramic acid (MurNAc), this biopolymer forms the layered peptidoglycan**.** Responsible for vegetative cell growth and division in Gram-positive organisms (41), cytosolic exo-acting GlcNAcases are known to be generated during peptidoglycan recycling (41)**.**

Previous work has shown that glycosyl triazoles is a novel class of anti-bacterial agent (1)**.** Glycosyl triazoles can be prepared through a 'click' reaction which matches galactosyl and glycosyl azides with an alkyne resulting in the inhibition of β- galactosidase and glycosidase (42)**.** Click chemistry relies on the ability to produce substances quickly through the connections of smaller units**.** Glycosyl amines have been known to inhibit a large variety of glycosidases by adding a positive charge on the active site which results in an interaction with carboxylate aspect**.**

Work recently reported by Kahn published in 2014 in the Medicinal Chemistry Communication, a Royal Society of Chemistry Journal, demonstrated growth inhibition of two species, ***B. subtillus and B. cereus***, in the micromolar range**.** These two inhibitors were created through an Ugi – reaction which resulted in 1-azido-*N*-acetylglucosamide and a plethora of terminal alkyne to be paired and tested for their ability to inhibit Gram-positive bacterial growth**.** Using one of the Ugi-derived GNT **B1.fgba**, inhibition of nitrophenol release was observed dependent of concentration when a synthetically- synthesized substrate β-*p*-nitrophenyl GlcNAc (*p*NP-GlcNAc) was in the presence of **B1.fgba** with full inhibition at 250μm (1)**.**

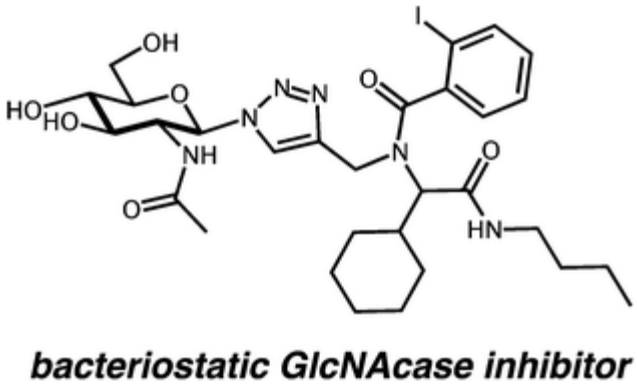


Figure 10: B1.fgba which is the lead compound that our other inhibitors have come from, the sugar is included as well (1).

METHODOLOGY

Getting to Know the Lab

Before beginning the Capstone research process I had to brush up on some simple techniques which would be critical throughout the entirety of the research. A sample of these techniques included:

1. Pipets
2. Autoclave
3. Fume Hood
4. Protein Gel

The pipets are used in order to precisely measure out a small aliquot of liquid that can then be used to create solutions. The autoclave on the other hand is a tool used to sterilize equipment utilizing extremely high pressure and heat. Both these tools are designed in order to promote sterility which is a major focus when working with molecules such as proteins and enzymes. Sterility allows researchers to ensure that the solutions and proteins they are utilizing are not contaminated by other molecules that may be arbitrarily found in the air, which is not being controlled for in experiments. When working with a procedure that requires sterility one will often work in what is referred to as the fume hood. The fume hood is able to function by diluting (sometimes hazardous) airborne material with air and drawing it through an exhaust

system which can bring the air outside of the building (43). This also protects the researcher as well as maintains a sterile environment to perform experiments that are easily contaminated (i.e. spreading agar plates).

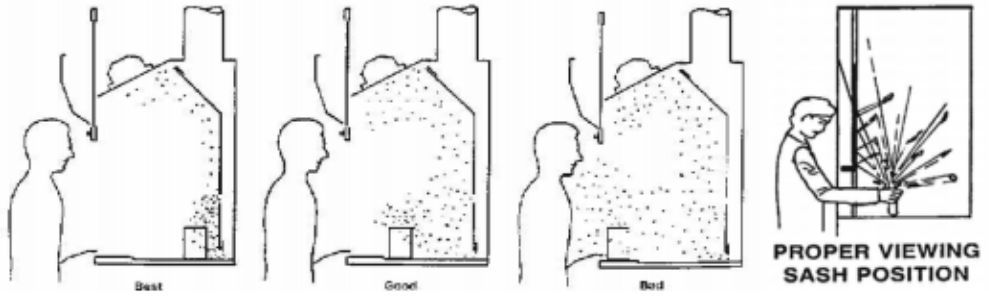


Figure 11: Proper position of the fume hood shield to prevent contamination and protect the researcher from potentially hazardous material (43).

Electrophoresis is the main principle behind the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) which allows for the separation of proteins utilizing an electric field. The SDS buffer is a detergent which denatures the samples which allows the gel to no longer be affected by protein shape. SDS also has a net negative charge (with an overall wide range of pH) which allows for the proteins to effectively all be charged negative. Upon use of SDS the proteins are able to be separated in the polyacrylamide gel solely by size and not shape nor charge. Since all of the samples have a net negative charge they are now attracted towards the positive anode which are at the bottom of the chamber (43). The larger molecules will have a more difficult time passing through the gel and thus won't be able to travel as far as the smaller molecules, which allows for the separation by size. After applying about 150 volts to the chamber for over an hour the samples begin to separate out revealing the molecular mass of protein, a defining quantitation. By comparing our samples to a standardized ladder we can easily assess the estimated molecular mass (in kiloDaltons).

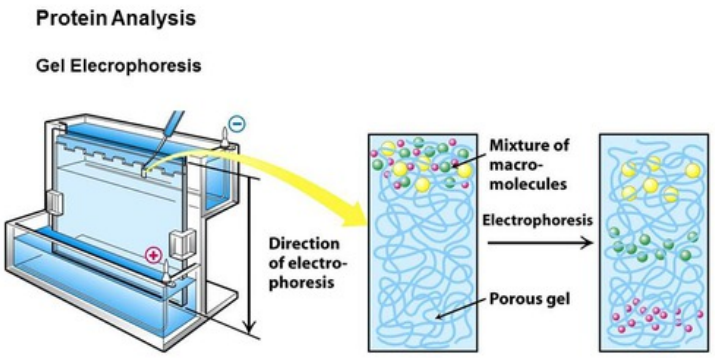


Figure 12: Depiction of the experimental technique of an SDS-PAGE gel (44).

Protein Purification

Before we could begin experiments that aimed to better understand the enzymatic activity and enzyme- substrate interaction we had to purify our protein of interest- LytG from *B. subtilis*. In order to purify our protein we had to first clone a 6xHis-tag residue onto the LytG protein and expressed it in *Escherichia coli* (*E. coli*). This will later allow us to exploit the 6xHis-tag to bind Nickel. Lysozyme and RNAase was added to a solution of Lysis Buffer (Appendix A) and our LytG pellets to lyse the cell wall and release our protein into solution. After brief incubation and sonication the cells were centrifuged for 15 minutes at 4ºC at 15 x *g*. This allows the heavy material from the cells to collect at the bottom and form a pellet, leaving the lighter material, including our LytG protein, in solution. To further purify our solution we passed it through a 2 μm filter to collect the lipids that we do not want to observe in our experiments.

After separation by weight and size Immobile Metal-Affinity Chromatography (IMAC) was used to exploit the unique affinity between Nickel and the 6xHis-tag residue. Two different buffer solutions were used to run this program of purification a binding buffer with a pH of about 8.0 and an elution buffer of a pH of about 4.5. These two buffers are each given a line that connects them to a gradient mixer which controls the pH that leaves into one line. This

one line is then connected to the column which contains our sample of somewhat purified protein as well as the Nickel resin which is able to bind to the Histidine residues on the LytG surface. When the gradient first starts the program we have a very high pH of 8.0 which allows for the Nickel resin to bind to the protein while the rest of the solution passes through and elutes off. After a few minutes the gradient mixer begins to slowly reduce the pH to a final pH of 4.5. This allows for the histidine residues on the surface of the protein to become protonated because it is becoming more acidic and allows for the protein to elute off of the Nickel resin column.

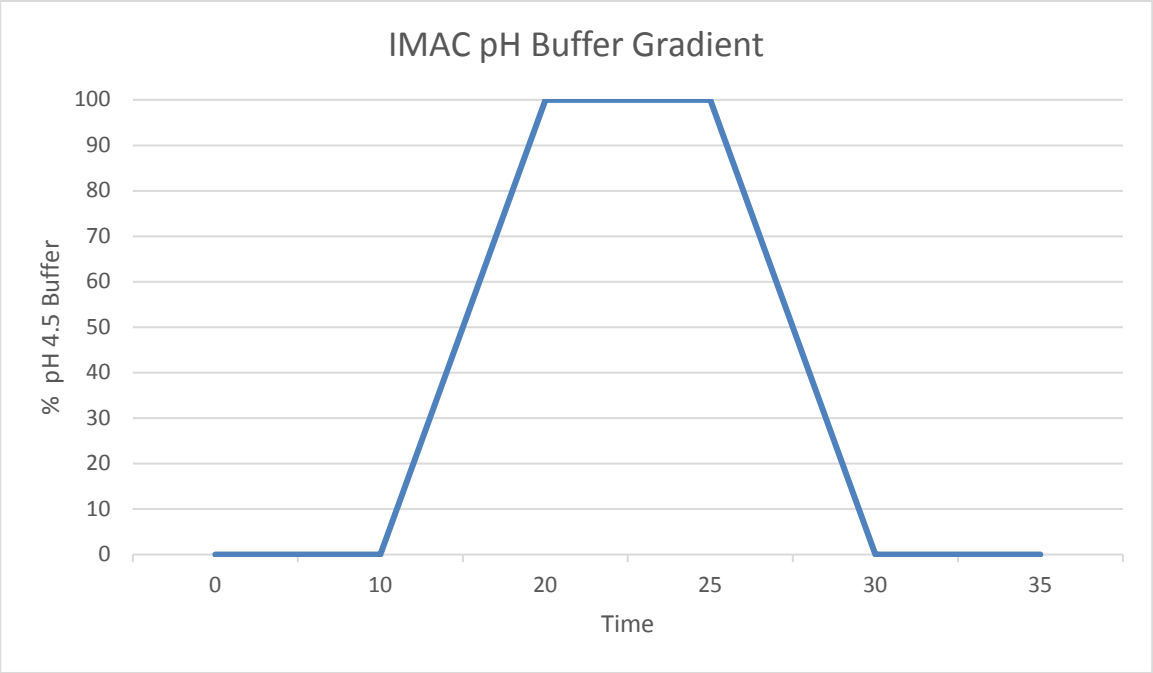


Figure 13: % pH 4.5 Buffer v. Time representation of the pH gradient of the IMAC gradient mixer (45).

The entire time this binding and elution is occurring a UV monitor is recording the absorbance of the sample, so as the protein elutes off of the column the UV monitor will record on the chromatogram what is referred to as a protein peak.

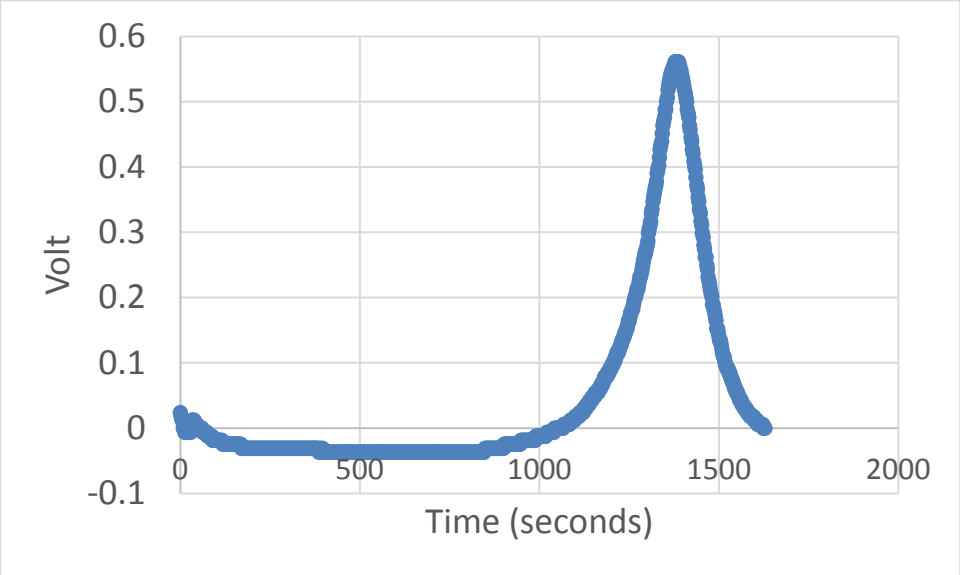


Figure 14: Chromatogram of LytG protein purification which identifies the start of the protein peak at about 1200 seconds and the end at about 1500 seconds (46).

Solution is being collected in what is known as a fraction collector which spins at 2 minutes a tube and allows for us to calculate which tube contains our protein. (In this chromatogram the protein peak starts at 1200 seconds, which divided by 120 seconds/ tube, is tube 10 and ends at 1500 seconds or tube 13. Therefore we would then collect the solution in tubes 10-13.

In order to find the concentration of the protein that we had in the purified solution of protein we had to utilize the unique characteristic of a protein which is the extinction coefficient. This is a value that is measured with a UV spec at 280nm and represents the ability of a protein to absorb light. For LytG the extinction coefficient is 1g/L = 1.227 Abs (0.1%). Therefore after we are able to measure a small 600µL sample at 280nm in the UV spec all we have to do is make a ratio to solve for concentration.

$$\frac{1\ \text{g/L}}{1.227\ \text{Abs}\ (0.1\%)} = \frac{X\ \text{g/L}}{0.352\ \text{Abs}\ (0.1\%)}$$

Scheme 1: Calculation of LytG protein concentration using 0.1% Abs at 280nm.

In this purification we were only able to get 0.2826 mg/mL of protein which was somewhat low, most often we were able to get at least 0.65mg/mL of protein with about 5mL of sample. This meant that on average we purified only about 3.25mg/mL of protein with each run and after about 7-10 days these samples would run off and no longer be viable for testing.

Turbidity Assay

After about a week of prepping and performing the purification we are able to start a turbidity assay which can, after several computations, tell us our Inhibitory Concentration 50 (IC50). This is the value at which our compound, **fgkc**, is able to inhibit 50% of the bacterial growth, which an industry standard to compare the efficiency of a spectrum of compounds. We used the UV spec which was used to determine the protein concentration again to perform these experiments. We utilized a standard of reaction buffer (Appendix B) to help cancel out superfluous noise that may be picked up and then compared it to our sample of **fgkc,** PG, reaction buffer and right before starting the spec, we add our LytG protein (APPENDIX C). This is a qualitative measure that aims to judge the ability of our inhibitors quickly, but it also allows us to establish a baseline. For each run we tested varying concentrations of our **fgkc** (95μM, 50 μM, 20 μM, 0 μM) as well as a Dimethyl Sulfoxide (DMSO) control which was expected to have zero inhibition. Each concentration was done in triplicates to account for human error as well as technical variations. Each individual sample took 15 minutes to analyze on the UV spec, which meant that each run took roughly four hours to perform plus the time to create each sample. The absorbance of each sample for the first 65-70 seconds is then taken at every 5 seconds which allows us to begin to create usable data that tells us about the effectiveness of our compound. First, the % initial OD is calculated for each time point by dividing the absorbance at the time point over the absorbance at T=0. These new values were then plotted on a graph of % initial OD vs. time and the slopes were recorded for each sample. The average slope of the DMSO control samples was then calculated, each samples slope was then divided by this average. This value was a measurement of % residual activity in these samples, once these averages were plotted on a graph we could use the equation of the line to solve for y=0.5 for the IC50.

Assay for Non-Specific Binding

Bovine Serum Albumin (BSA) is a protein that is found in cattle and is used in assay to assess an inhibitors specificity against proteins. BSA is considered a "sticky" protein in terms of its affinity for inhibitors and other molecules, which makes it an ideal candidate to test if our compounds are specifically inhibiting LytG or if they are working by non-specifically killing, which would make it a less "druggable" compound. The BSA assays were set up in 96 well plates and, in technical duplicates, varied both concentration of the BSA protein as well as the concentration of the **fgkc** which allowed us to assess the impact of these varying concentrations on bacterial growth (Appendix D). We had a row of control which contained DMSO, Luria Broth media and BSA. Every other well contained a different amount of **fgkc**, Luria Broth media, BSA protein, and then as soon as all of the wells were filled (about 400 pipet tips later) we added our *B. subtilis* inoculum and allowed three hours of incubation (Appendix E). After the incubation period we added a final compound to each well, Remazol which changes color when in the presence of redox reactions which occurs when bacteria is able to grow. At first all of the wells are a deep purple, but after about 15-20 minutes the color begins to change, most prominently in our control well, which we expected bacterial growth in.

Saturation Transfer Difference –Nuclear Magnetic Resonance

Saturation transfer difference nuclear magnetic resonance (STD-NMR) is a tool used to characterize the interaction between our protein of interest, LytG and the ligand, our diamide inhibitors. This information can later be used to better understand what aspects of our inhibitors are the characteristics that confer the inhibitory activity and potency to the molecule. Currently an undergraduate student at Brown University is working to adapt protocols and conditions from a paper, "Ligand-Receptor Binding Affinities from Saturation Transfer Difference Nuclear Magnetic Resonance Spectroscopy: The Binding Isotherm of STD Initial Growth Rates" (47) using L-tryptophan and BSA to work for our LytG and diamide inhibitors.

STD-NMR utilizes an extremely strong magnetic field, stronger than the Earth's gravitational pull, which aligns all of the hydrogen atoms poles with the pole of the giant magnet. These hydrogen act as small bar magnets within the overarching larger magnetic field, thus their

north poles align with the giant magnet's north pole and south with south. After all of the hydrogens are properly lined up we apply a specific frequency of FM radiation to our protein LytG which is a form of high energy. The FM radiation is able to impart high energy onto the hydrogen atoms which in turn reverses the poles of these theoretical bar magnets, thus the north poles on the hydrogens are now aligned with the south pole of the giant magnetic field. Next we introduce our diamide inhibitor to our LytG protein which naturally interacts. When the diamide inhibitor begins to interact with the high energy hydrogen atoms on the protein, the hydrogen atoms on the diamide inhibitor that most closely interact with the protein gain protein's high energy. This forces the hydrogen atoms on the diamide inhibitor that are most closely reacting with the protein to flip poles as well (their north is now aligned with the magnetic field's south). Since this is a high energy state we are able to monitor the fall of energy these hydrogen atoms (on the diamide inhibitor) experience which emits a signal (48). This data is crucial in determining which aspects of our inhibitor are most important in the LytG interaction. Since the paper we are adapting the protocol from utilizes L-Tryptophan and BSA, the conditions for LytG and our diamide inhibitors are slightly different. Some of the parameters this student is working on is the length of time the FM radiation should be applied to our protein, at what intensity of FM radiation should we hit our protein and, finally, how long our protein should be able to adjust to the high energy state. Soon we hope to identify the correct parameters that give us data that helps to elucidate the LytG- diamide inhibitor interaction.

RESULTS

Protein Purification

In order to ensure that our purification method was successful in isolating our protein of interest we ran an SDS-PAGE gel. By separating our sample out by size and comparing it to a standardized ladder we will be able to confirm if the protein we have purified is the correct molecular mass (about 45kDa). Figure 15 depicts the gel after our first purification protocol, which confirms the presence of solely our protein of interest.

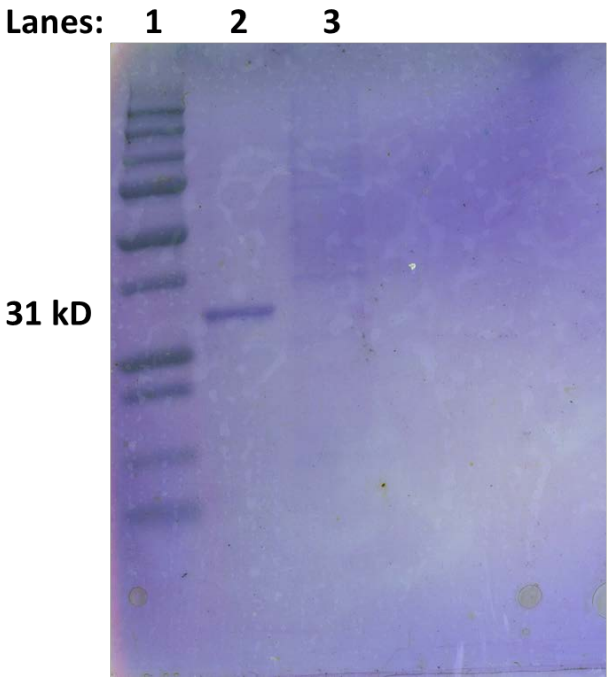


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Figure 15: Depiction of an SDS-PAGE gel which confirms the presence of LytG after purification immobilized metal-affinity chromatography (Broad Range). Lane 1 is the BioRad Standard Protein Ladder, Lane 2 is our LytG purified protein and Lane 3 is our affinity column flow through which contains some membrane proteins which can be seen (49).

Turbidity Assay

After the first two trials of turbidity assay we were still getting data that indicated an IC50 in the hundreds which we recognized to be somewhat inconsistent with the rest of the data we had collected to date. We did a little investigating and realized that the **fgkc** compound that was a crucial aspect of the assay composition had degraded thus the enzyme was no longer active. We have since been able to run the assay once more with new compound and have gathered data that is much more aligned with the rest of our data. Currently our IC50 is at 17.8µM, which is still higher than we expected but that may be due to technical error since the protocol requires such precision. We plan to iron out a few more trials to make sure that our data is correct.

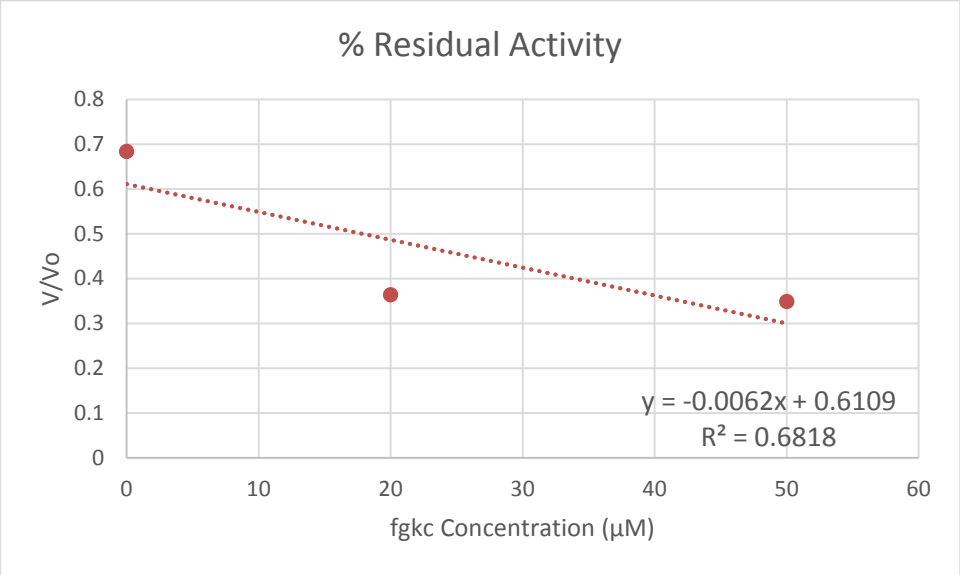

*Figure 16: The % residual activity with our **fgkc** compound resulting from the turbidity assay (50).*

Assay for Non-Specific Binding

BSA assays are inherently qualitative which requires the use of human estimation to transform them into graphical representations. The concentration of BSA is plotted on the x-axis and the apparent minimum inhibitory concentration (MIC) is plotted on the y-axis. This y-axis value is derived from looking at the 96 well plates and identifying at what **fgkc** concentration does the color change from purple, to pink- indicating bacterial growth.

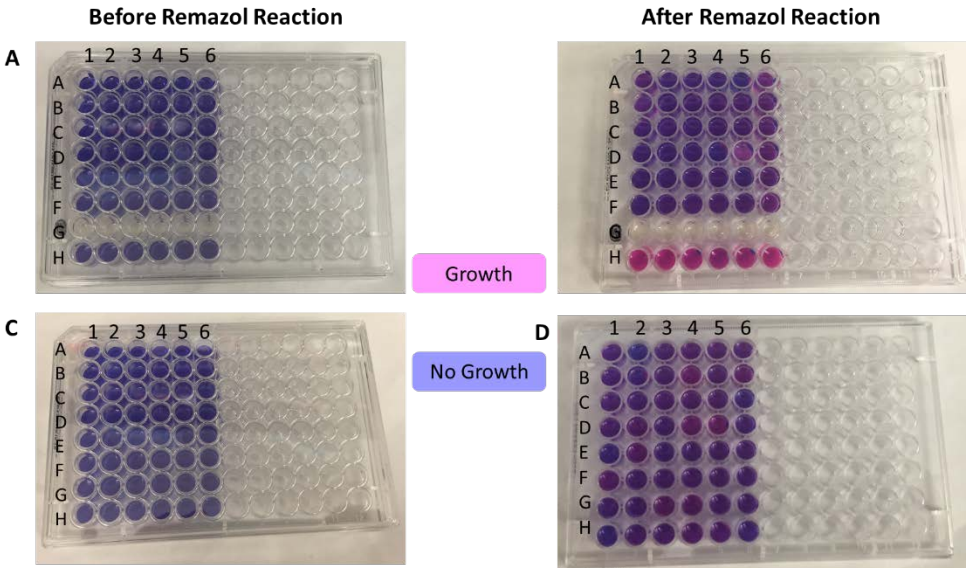

Figure 17: Before the Remazol color agent is allowed to incubate for 15 -20 minutes all of the wells remain purple. After this period the wells that had bacterial growth begin to turn purple (51) Please refer to Appendix D.

For almost every concentration of BSA the apparent MIC was at the lowest concentration of **fgkc** was the 1/128 concentration of 0.107mg/mL, however since this was the lowest concentration we could not see specifics from concentrations around that range. Thus, in the future we plan to run a subsequent BSA assay with concentrations of fgkc closer to the range of 0.107mg/mL instead of higher concentrations.

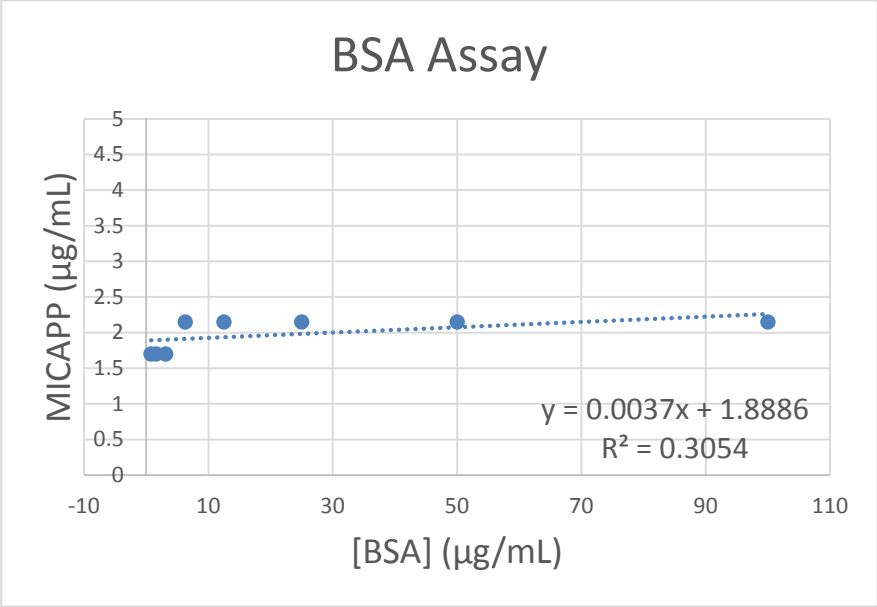

Figure 18: The concentration of BSA was plotted versus the apparent MIC which yielded an almost horizontal line indicated that our inhibitor binds specifically. If the slope had been steep it would have indicated non-specific binding (52).

DISCUSSION

This research is considered discovery science in that we have created a library of aglycone compounds which may or may not exhibit inhibitory characteristics and have slowly twiddled down the list to potentially lethal targets. Our two best compounds to date have remained **fgkc** and **fgka**, which we plan to continue researching way to make them more potent and effective. Some characteristics have already been identified to understand what makes these two compounds efficient while others are not. One of these features is the presence of an Iodine atom off of the aglycone, we hope to identify more through the use of the STD-NMR. Previous research using Triton detergent on these compounds have also supported the notion that these compounds do not work by simply aggregate onto the surface of proteins to kill them, and paired with the specific binding results of the BSA assay these compounds continue to look promising.

CONCLUSION

Moving forward we plan to keep working towards biochemically characterizing these interactions so that we may gain a better perspective of what makes these compounds effective inhibitors. In doing so we must re-do the turbidity assays so that we can get a clearer picture of the inhibitory activity. We also must repeat another trial of the BSA assay so that we can pin point the MIC with confidence rather than a rough estimate. We also hope to produce some STD-NMR data soon to gain further insight. Finally, one of the bigger issues was that our compounds are remaining water insoluble meaning that they cannot dissolve in water which makes experiments down the road much more tenuous and difficult so we are hoping to modify that characteristic.

APPENDICES

Appendix A – (Lysis Buffer)

50mM Na_2HPO_4

400mM NaCl

10% Glycerol

pH 8.0

Appendix B – (IMAC Buffers)

Reaction Buffer:

10mM Sodium Citrate

10mM Magnesium Chloride

0.01% Triton X-100

Elution Buffer:

50mM Na_2HPO_4

400mM NaCl

10% Glycerol

pH 4.5

Appendix C – (Turbidity Assay Protocol)

25mL lysis buffer for 3 pellets, keep on ice and add 10 μL lysozyme and miniscule amount of RNAase. Sonicate for 4 minutes (10s on/ 20s off 60% Intensity). Centrifuge at 10,000 rpm for 15 minutes at 4ºC and pass supernatant through a 2μm filter.

Add 1mL of Nickel resin to Eppendorf and centrifuge briefly several times adding reaction buffer between each cycle.

Load Nickel Resin on to column and allow to incubate overnight.

Load sample onto column slowly and follow with 2 mL of pH 8.0 buffer.

Purge both lines with water to get rid of air bubbles and ethanol which could form salts, for several minutes.
Run with 100% pH 8.0 buffer for 3 minutes
Run program and run fraction collector simultaneously while watching for protein peak on the chromatogram.

Appendix D – (BSA 96-Well Plate set-up)

	PLATE 1	1/4 [fgkc]↓	1/8 [fgkc]↓	1/16 [fgkc]↓	1/32 [fgkc]↓	1/64 [fgkc]↓	1/128 [fgkc]↓
		1	2	3	4	5	6
1/2 BSA→	A						
1/2 BSA→	B						
1/4 BSA→	C						
1/4 BSA→	D						
1/8 BSA→	E						
1/8 BSA→	F						
	G						
CONTROL→	H						

	PLATE 2	1/4 [fgkc]↓	1/8 [fgkc]↓	1/16 [fgkc]↓	1/32 [fgkc]↓	1/64 [fgkc]↓	1/128 [fgkc]↓
		1	2	3	4	5	6
1/16 BSA→	A						
1/16 BSA→	B						
1/32 BSA→	C						
1/32 BSA→	D						
1/64 BSA→	E						
1/64 BSA→	F						
1/128 BSA→	G						
1/128 BSA→	H						

Appendix E – (BSA Protocol)

Each well contains:

- 2 µL BSA
- 2 µL **fgkc**
- 91 µL LB Media
- 5 µL inoculum (1/20 dilution of OD_{600}=1.0)

Control contains:

- 2 µL DMSO
- 93 µL LB Media
- 5 µL inoculum

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